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SYNTHESIS OF BIOTINYLATED FERROCENE, AND ITS APPLICATION AS A REDOX PROBE FOR ELECTROCHEMICAL IMMUNOSENSOR

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ABSTRACT

The coupling compound of Biotin (Vitamin H) and redox molecule ferrocene through covalent bonding was studied. Synthesis and structure identification of Biotinylated 1 - (- 2-aminoethyl) ferrocene were performed by ^{1}H-NMR, ^{13}C-NMR and MS. The ultimate goal of this paper is to develop derivatives of ferroceneservingas an electrochemical probe that would enhance the performance of immunosensor.An immunosensor based on polypyrrole-streptavidin layer as platform for immobilization of biotinylatedferrocene and antibodies was developed. The measurement of redox signal of ferrocene was analyzed by differential pulse voltammetry (DPV) method and underlined a variation of redox properties upon the antigen interaction. The introduction of the redox biotinylatedferrocene into streptavidin-polypyrrole layer affords a good sensitivity of the biosensor with a detection limit of 0.16 pg.mL^{-1}.

*Keywords:*immunosensor, ferrocene, redox probe, electrochemical detection, polypyrrole.

1. INTRODUCTION

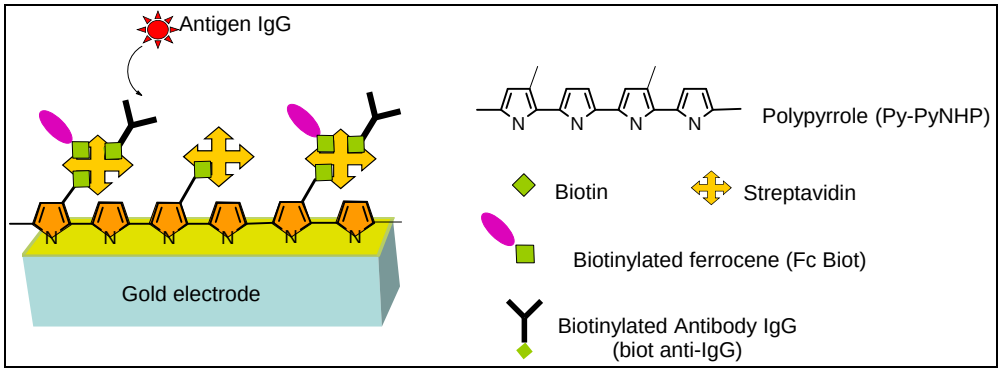
In recent years, electrochemical immunosensor has been studied and proven to be potential devices in many field such as clinical field [1], medicine and environment controlling, food safety [2]. These devices have many advantages such as high endurance, low cost, sensitivity and high selectivity, compact size, portability, simple manufacture process. The biosensors allow detecting biological molecules which are of small quantity, small surface area, and low concentration. Immunosensor is usually produced by immobilizing antibody on electrode surface, which can rapidly detect antigens through specific antibody /antigen interaction.

The electrochemical immunosensor essentially based on oxidation -reduction reaction of conductive materials such as carbon nanotubes [3], nanoparticles [4], semiconductive polymer

such as polypyrrole, polyaniline, polythiophene [5,6,7]. Antibody / antigen interaction is monitored by electrochemical methods such as cyclic voltammetry, differential pulse voltammetry, based on oxidation -reduction reaction of these materials. Among the semiconductive polymer, polypyrrole is used in several studies of electrochemical sensor manufacture because of biological compatibility, stability in water. To improve the sensitivity of sensor, the electrochemical indicator combined with polymers has been used such as $K_4Fe(CN)_6$ / $K_3Fe(CN)_6$ [8], viologen [9], ferrocene. In particular, Ferrocene is used like oxidation - reduction molecules in the electrochemical immunosensors because of the high electrochemical activity [10].

There are many methods to immobilize antibodies on the surface of the electrode as physical adsorption [11], covalent bond [12] or biotin / streptavidin bond [13, 14]. Physical adsorption has the advantage of manufacture but the bond is easilybreakable because of its weakness. Covalent bond has the advantage of high endurance but difficult to synthesize. Besides, Streptavidin has four binding sites for biotin in molecular structure capable of detecting biotin molecules by specific link with high affinity (Ka = 1015M-1) [15].

The purpose of this project was to synthesize Biotinylated 1 – (-2- aminoethyl) Ferrocene (Fc Biot) that will serve as an electrochemical probe to enhance the conductive properties of the immunosensor. The structure of Fc Biot compound was determined by ^{1}H-NMR spectrum, ^{13}C-NMR and Mass Spectrometry. After that, the group studied the constructionof electrochemical immunosensor based on Fc Biot compound in combination with biotin / streptavidin interaction, using polypyrrole polymer in order to rapidly detect antigen (Scheme 1). The achieved resultsopen the way to the development of electrochemicalmultisensor systems.



Scheme 1. Immunosensor construction.

2. MATERIALS AND METHODS

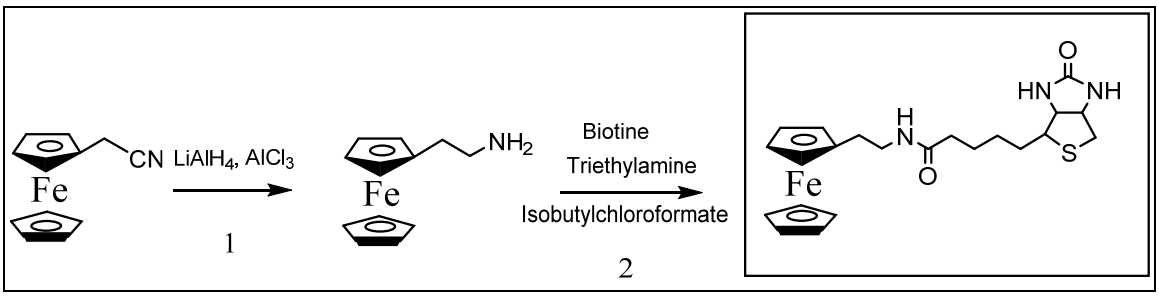
2.1. Materials

Pyrrole (py) was purchased from Sigma-Aldrich and distilled under argon before using. Biotin, streptavidin, biotinylated antibody IgG (biot anti-IgG), antigen IgGand Phosphate Buffer Saline (PBS) tablets, were purchased from Sigma-Aldrich.

Etan nitrile ferrocenyl, LiAlH4, AlCl3, Trietylamine, Isobutylchloroformate, DMF, THF were purchased from Sigma-Aldrich.

2.2. Synthesis

The Biotinylated 1 - (- 2-aminoethyl) ferrocene (Fc Biot) was synthesized frometan nitrile ferrocenylas shown in Scheme 2.



Scheme 2. Synthesis of Biotinylated 1 - (- 2-aminoethyl) ferrocene(FcBiot).

2.2.1. Synthesis of 1 - (- 2-aminoethyl) ferrocene (Fc NH_2), first step as seen in Scheme 2

Under argon atmosphere, $AlCl_3$ (0.58 g, 4.4 mmol) was addedin9 mL of THF and stirred at 0 °C for 5 min. $LiAlH_4$ (0.87 g, 23 mmol) was added to this mixture and the reaction was stirred for 15 min. Then the solution of 1-(-2-cyanoethyl) ferrocene (1 g, 4.4 mmol) in 10 mL of THF was added drop wise and the reaction was warmed to room temperature and mixed during 3 hours. The contents of the flask were evaporated using a rotary evaporator. The residue was dissolved in water and extracted several times with ether. The collected organic layers were washed with water and with a saturated solution of NaCl, dried over $MgSO_4$ and concentrated in vacuum. 0.87 g of orange oil were obtained with yields of 86%.

2.2.2. Synthesis of 1 - (- 2-aminoethyl) ferrocene (Fc Biot), second step as seen in Scheme 2

Biotin(5 g; 2.05 mmol) and triethylamine (3 mL; 2.05 mmol) were reacted with the isobutylchloroformate (2 mL;2.05 mmol) diluted in 25 mL of DMF under argon at -15°C. The reaction was stirred for 30 min. Then a solutionof 1-(-2-aminoethyl) ferrocene and triethylamine(3 mL; 2.18 mL) in 25 mL of DMF was added drop wiseto the mixture at -15°C and stirred for 15 min. The reaction was then mixedduring 12 hours at room temperature under argon. Thesolvent was evaporated and the residue was extracted with dichloromethane, dried over $MgSO_4$, filtered and concentrated.The product was purifiedby chromatography over silica gel using dichloromethane/methanol 98/2. 0.2 g of yellow crystals was obtainedwith the yield of 22 %.

2.3. Instrumentation

Electrochemical polymerization and characterization were performed using a potentiostatgalvanostatautolab PGSTAT 30 controlled by GPES software. The three electrode cell was purchased from Basi and consists of a platinum mesh as a counter electrode, gold disc (surface 0.0201 cm^{-2}) as working electrode and Ag/AgCl as reference electrode.

2.4. Electropolymerization of copolymer Py-PyNHP

The copolymer film poly[pyrrole-3-N-(hydroxyphthalimido) pyrrole] (Py-PyNHP) was deposited on the gold electrode, in a electrochemical cell containing a 10 mM solution of pyrrole (Py) and pyrrole functionalized by an active ester (Py-NHP) in a ratio of 8 :2 and 0.5 M $LiClO_4$ in acetonitrile. The film was grown by cycling the potential from -0.4 to 1.2 vs. Ag/AgCl with

the scan rate of 100 $mV.s^{-1}$. The reaction was stopped after 10 cycles corresponding to a maximal current of 11 mA at the potential 0.29 V vs. Ag/AgCl.

2.5. Construction of the Biosensor

The first step of immunosensorconstruction was the covalent bonding of biotin hydrazide to the copolymer Py-PyNHP (Scheme 1), performed by the deposition of 40 mL of a 2 $mg.mL^{-1}$ solution of biotin hydrazide in PBS buffer pH 7.4 for 30 min at room temperature. The resulting biotinylatedpolypyrrole film was washed with distilled water and PBS buffer.

The second step was the recognition of biotin and 40 mL of a 100 $\mu g.mL^{-1}$ streptavidin in PBS buffer pH 7.4 for 30 min. Afterwards, 40 mL of 2 M biotinylatedferrocene solution was incubated during 2 hours at room temperature. After, 40 mL of 8 $mg.mL^{-1}$biotinylated antibody anti-IgG solution in PBS was incubated during30 min at room temperature. After each immobilization step, the modified surface was washed with distilled water and PBS buffer, to eliminate non-bonded molecules on the copolypyrrole surface. Before antigen detection, the surface was blocked with 40 mL of 50 $mg.mL^{-1}$ of casein solution during 30 min at room temperature to avoid the non-specific interactions on the copolypyrrole surface. After each step of immunosensor construction, the surface modifications were controlled by CV and DPV methods.

2.6. Antigen detection

The incubation was performed by immersing the modified electrode in each solution of antigen (1 $pg.mL^{-1}$; 10$pg.mL^{-1}$; 100 $pg.mL^{-1}$; 1 $ng.mL^{-1}$) during 30 min at room temperature.

3. RESULTS AND DISCUSSION

3.1. Determination of the structure and electrochemical properties of Fc Biotcompound

3.1.1. Structure determination of Fc Biot compound

The coupling of amino group and biotin was performed in presence of isobutyl chloroformate and triethylamine to afford after purification the biotinylatedferrocene derivative (Scheme 2).The structure of compound Fc Biot was characterized by ^{1}H-NMR, ^{13}C-NMR and mass spectrometry (Table 1).

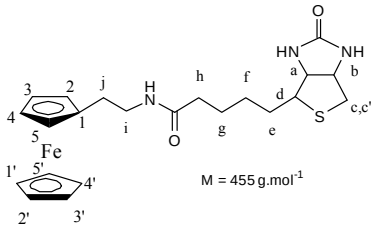
A^{1}H-NMR spectrum and ^{13}C-NMR showed characteristics spectrums of ferrocene group, appear at δ 4.1 ppm, corresponding to the cyclopentadienyl rings proton $CH_{2,3,4,5}$and$CH_{1',2',3',4',5'}$.

A ^{1}H-NMR spectrum of Fc Biotcompound showed two triplets at δ 2.2 ppm and δ 2.55 ppm corresponding to CH_{2h} and CH_{2j} . Others NMR signals typically of biotin were observed atδ = 3.22 - 4.5 ppm and δ = 2.74 - 2.96 ppm, corresponding to $CH_{a,b,d}$and $CH_{c,c'}$.

The presence of two carbon CO and CONH at δ = 164.7 – 174.5 ppm in the ^{13}C-NMR approved the coupling with biotin.The analysis result of electrospray ionisation mass spectrometry showed the molecular mass of Fc Biot compound.

The NMR spectra and mass spectrometry utterly confirm the structure of Fc Biot with molecular formula $C_{22}H_{30}FeN_3O_2S$ and the expected structural formula as shown in Table 1.

Table 1.[1]H-NMR and [13]C-NMR chemical shifts and Mass Spectrometry of Fc Biot compound.

Compound	 Biotinylated 1 - (- 2-aminoethyl) ferrocene	
Mass spectrometry (M+H$^{+)}$	456	
Chemical shift	**δ ^{1}H (ppm)**	**δ ^{13}C (ppm)**
CH_1		85.6
CH_2,CH_3, CH_4, CH_5	4.1	68.2
$CH_{1'}$,$CH_{2'}$, $CH_{3'}$,$CH_{4'}$, $CH_{5'}$	4.07	67.9
CH_{2j}	2.55	35.5
CH_{2i}	3.4	67.1
CH_{2h}	2.2	29.1
$CH_{2g,\ 2e}$	1.67	28.1
CH_{2f}	1.44	25.5
CH_d	3.22	55.5
$CH_{c,\ c'}$	2.74	39.7
$CH_{a,b}$	4.5	60.2
CO		164.7
CONH		174.5

3.1.2. Electrochemical properties of Fc Biot compound

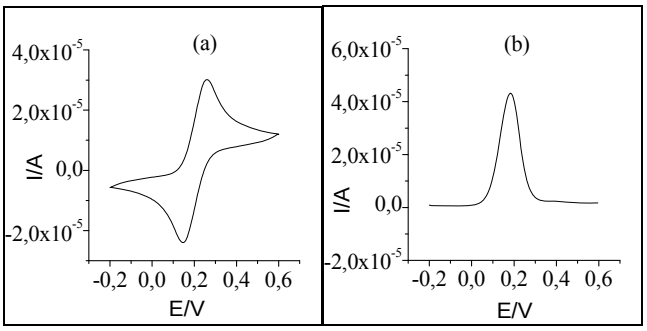


Figure 1. Cyclic voltammogram (a), DPV (b) of Fc Biot compound 10 mM in PBS buffer 7.4, scan rate 50 mV.s^{-1}.

The electrochemical characterization of the Fc Biot compound was achieved in 10 mM PBS buffer pH 7.4 by cyclic voltammetry and DPV. Figure 1(a) exhibited one anodic peak at 0.2 V in reverse scan, assigned to the electrochemical reaction: $Fe^{3+} + e^{-} = Fe^{2+}$. These redox properties were typically of ferrocene [16,17]. The DPV curve of Fc Biotpresented the same anodic wave at 0.2 V as observed in CV curve (Fig. 1 (b)).

3.2. Immunosensor construction

The first step of the immunosensor construction was performed by the electropolymerization of two monomers pyrrole (Py) and pyrrole bearing an activated ester group 3-N-(hydroxyphthalimido) pyrrole (Py-NHP) in a ratio of 8:2, formed a conducting copolymer film on a gold electrode surface. The polymerization process was realized by cyclic voltammetry in acetonitrile from -0.4 V to 1.2 V vs. Ag/AgCl. The voltammogram shows a current at around 0.2 V that increases after each cycle, demonstrating the polypyrrole formation and deposition onto the electrode surface.Biotin hydrazide was then attached on the surface through covalent reaction leading to amido link followed by the immobilization of streptravidin to form polypyrrole-streptavidin layer. All these steps were carefully characterized and optimized in our previous work [14].

DPV of redox probes on the polypyrrole-streptavidin surface displays two oxidative peaks, an intensive one at 0.18 V and a smaller one at 0.48 V which increases during biosensor construction (Fig. 2). The intensive one is assigned to both the oxidative reaction of ppy to ppy^{+} and Fc to Fc^{+} immobilized on the streptavidin layer. Besides, the broad wave at 0.18 V can be explained by the random distribution of ferrocene sites on the polypyrrole-streptavidin film contributing to different non-equivalent redox sites on the coated electrode. The anodic wave at 0.48 V results certainly from the oxidation of another ferrocenyl species, less electroactive, that becomes predominant after biomolecules immobilization [18,19].

The successive immobilization steps of the antibody anti-IgG on the modified polypyrrole layer were performed by incubation of the modified electrode with biotinylated followed by casein, used to block the surface in order to avoid any proteins adsorption onto the copolymer film during the antigen detection. Fig. 2shows the decrease of oxidation currents at both 0.18 V and 0.48 V after each addition of biomolecules (antibody and casein) on the surface. In the two cases, this phenomenon may be explained by the modifications of the polypyrrole layer by proteins that block charge transport and penetration of counterions to assure the redox process.

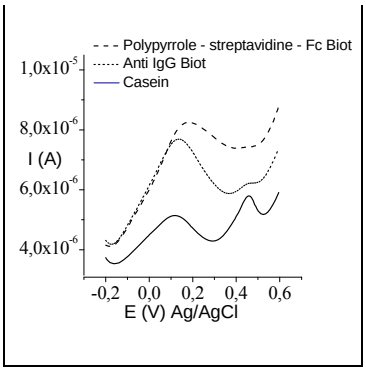


Figure 2. DPV analysis of copolymer surface modified Fc Biot, antibodies Anti IgGBiot and casein in 10 mM PBS 7.4, scan rate 50 $mV.s^{-1}$.

3.3. Antigen detection

The biosensor is incubated with successive addition of various concentrations of antigen IgG in PBS 10 mM pH 7.4.The oxidation currents at both 0.18 and 0.45 V decrease since the addition of 1 pg.mL^{-1} and reach the saturation at 100 pg.mL^{-1} (Fig.3(a)). The variation of peak current is directly proportional to the antigen-antibody complex formation. The decrease in current intensity is explained by the formation of antigen-antibody complex, which leads to a slow penetration of ions and avoids the electron transfer to electrode [20].

The stability of biosensor was performed by storing the biosensor at various times and testing the response before antigen detection. This was realized during two days and the signal remains the same. The signal variation is obtained only when the antigen was attached on the surface. Results demonstrated an important stability of the electrochemical signal of ferrocene within the time. Reproducibility was studied with five independent measurements at various concentration levels and relative standard deviation (RSD) of 5% was evaluated. The RSD value is an average of the values obtained at each of these concentrations.

Linear variation of current versus concentration of antigen was measured from 1 pg.mL^{-1} to 10 pg.mL^{-1} for ferrocene redox probe and a detection limit was estimated at 0.16 pg.mL^{-1} (Fig. 3(b)). The successful introduction of redox probe Fc Biot was enhanced the performance of the immunosensor in terms of sensitivity, compared to other immunosensors using electrochemical indicators ANTA / Cu or hydroquinone [21, 14].

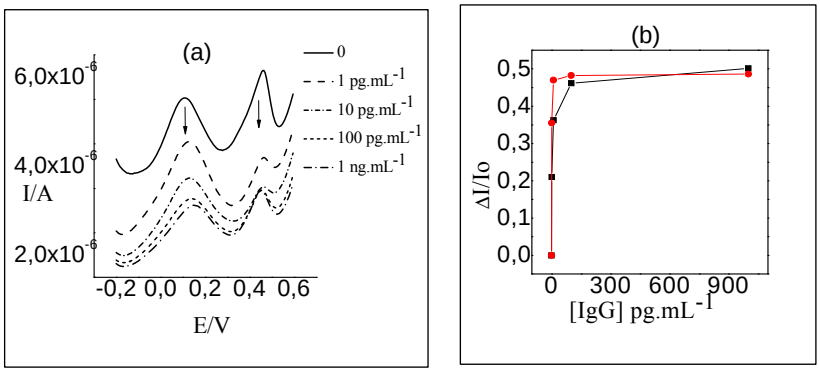


Figure 3. (a)DVP analysis of surface modified with biotinylatedferrocene after interaction of different concentration of IgG (100 pg.mL-1–1 mg.mL-1) in PBS 10 mM pH 7.4, (b) Calibration curve.

4. CONCLUSIONS

In this study, we have successfully synthesized the Biotinylated 1 - (- 2-aminoethyl) ferrocene (Fc Biot) compound and characterized by ^{1}H-NMR, ^{13}C-NMR and mass spectrometry. The second goal of the project, electrochemical characterization of the Fc Biot was achieved. Afterwards, an immunosensor based on polypyrrole layer as platform for immobilization of biotinylatedferrocene was developed. We demonstrated that the introduction of the redox probe into streptavidin-polypyrrole layer affords a good sensitivity of the biosensor with a detection limit of 0.16 pg.mL^{-1} for ferrocene. These studyresults demonstrate that this approach is suitable for the immobilization of various biotinylated redox molecules and the formation of multi-electrochemical system detection. The versatility of redox molecules with various oxidation potential and the easy immobilization of such redox molecules on polypyrrole-streptavidin layer open the way for multiplexing electrochemical detection array.

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TÓM TẮT

TỔNG HỢP BIOTINYL - FERROCENE VÀ ỨNG DỤNG LÀM ĐIỆN CỰC OXY HOÁ KHỬ CHO CẢM BIẾN SINH HỌC ĐIỆN HÓA

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Hợp chất liên kết Biotin (Vitamin H) với phân tử chất oxy hoá khử ferrocene thông qua mối liên kết cộng hóa trị đã được nghiên cứu. Việc tổng hợp và xác định cấu trúc Biotinyl1 - (-2-aminoethyl) ferrocene đã được thực hiện nhờ kỹ thuật cộng hưởng từ ^{1}H-NMR, ^{13}C-NMR và khối phổ MS. Mục tiêu của bài báo này là phát triển các dẫn xuất của ferrocene làm điện cực điện hoá có thể thực hiện các chức năng của cảm biến sinh học. Một cảm biến sinh họcdựa trên

lớp polypyrrole-streptavidin làmcơ sở để cố định biotinyl- ferrocene và chất kháng thể đã được phát triển. Kết quả đo tín hiệu oxy hoá khử của ferrocene được phân tích bằng phương pháp xung điện thế vi phân (DPV) và chỉ ra sự khác biệt giữa tính chất oxy hoá khử và sự tương tác của kháng nguyên. Việc đưa biotinyl -ferrocene có tính oxy hoá khử vào lớp streptavidin-polypyrrole đã giúp đạt đến độ nhạy cao của cảm biến sinh học, đến giới hạn phát hiện 0,16 $pg.mL^{-1}$

*Từ khóa:*cảm biến miễn dịch, ferrocene, điện cực khử, phát hiện bằng điện hóa, polypyrrole.